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14. ABSTRACT

Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for Dachsous 2 (Ds2) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years (p= 1.1 x 10-5 by students t-test). This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer. Ds2 encodes a large cell adhesion molecule implicated in planar polarity and cell proliferation, To determine the significance of the increased levels of Ds2 in recurrence we will: 1) Validate gene expression array results and determine if Ds2 proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon Ds2 overexpression. 3) Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in the mouse

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INTRODUCTION:

Microarray analysis of tumors women with axillary node negative (ANN) breast cancer revealed that a cDNA for *Dachsous 2* (*Ds2*) is one of the most highly discriminatory genes in tumors from patients who had experienced early recurrence, compared to tumors from patients who did not recur for at least ten years (p= 1.1 x 10-5 by students t-test). *This suggests that increased transcription of Ds2 may be a predictive indicator of recurrence in ANN breast cancer. Ds2* encodes a large cell adhesion molecule[1]. The *Drosophila* homolog of *Ds2*, *Ds,* functions as a planar cell polarity (PCP) ligand for the large cadherin Fat[2-5], and together Ds and Fat regulate tissue organization through a PCP signaling pathway. Recent data has also linked Ds with a newly described growth control pathway, the Hippo kinase pathway[6-9]. To determine the significance of the increased levels of *Ds2* in recurrence we will: 1) Validate gene expression array results and determine if Ds2 proteins levels increase in tumors 2) Determine if PCP or Hippo pathway gene expression is altered upon Ds2 overexpression. 3) Determine the effects of altering Ds2 levels on proliferation and tumor susceptibility in the mouse

BODY:

Task 1. ***Determine if Ds2 protein and transcript levels are increased in tumors from patients that have recurrent breast cancer.***

A) Generate anti-Ds2 antibody. Our first goal was to generate specific antibodies to Ds2 that could be used in paraffin section analysis of tumour samples from ANN patients.

Fig. 1

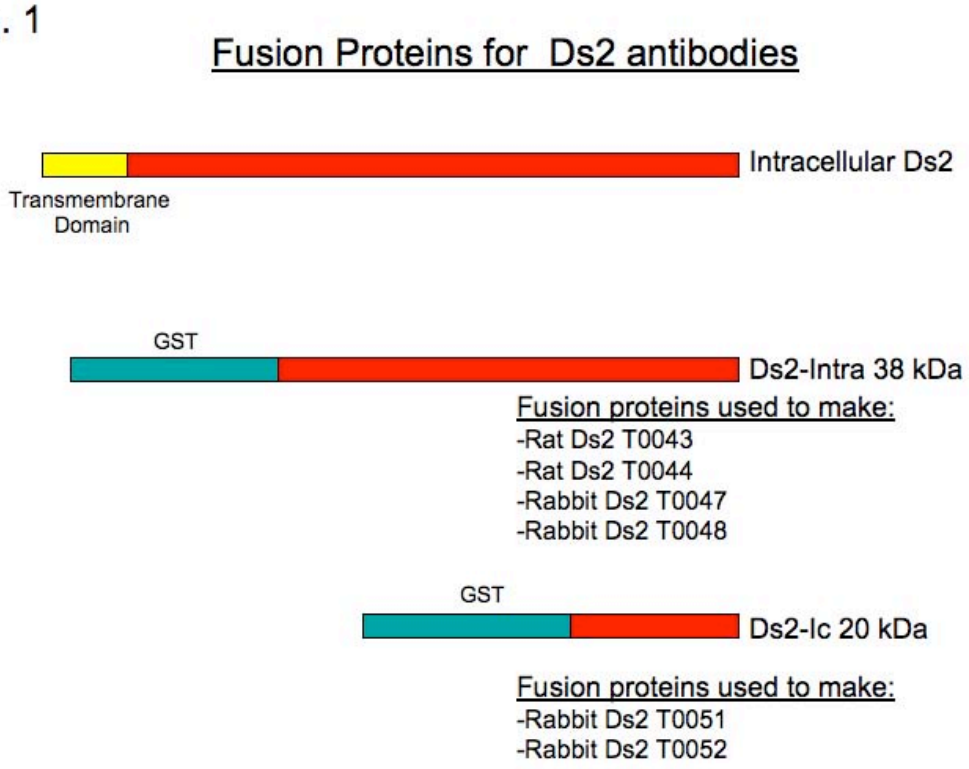

Figure 1. Constructs used to generate fusion proteins for specific antibodies to Ds2. Green indicates the GST portion, and red the segment of the intracellular domain of Ds2 used to generate antibodies.

We initially generated 4 antisera to the entire cytoplasmic domain of Ds2, immunizing 2 rats and 2 rabbits (antibodies TO43, 44, 47 and 48: **Figure 1**). We chose to assay the antibodies using an immunohistochemistry of mouse hippocampus, as it has a simple and clear expression pattern of Ds2[1]. None of these antibodies showed any clear expression pattern in the hippocampus assay, despite trying a large number of staining conditions (we assayed cryosections and paraffin sections, tried a large dilution series, different blocking and different antigen retrieval approaches) (data not shown). We then generated fusion proteins to smaller portions of the cytoplasmic domain and inoculated 2 rabbits with this antigen (antibodies TO51 and TO52: **Figure 1**). These antibodies showed high specificity in both cryosection analysis and paraffin analysis of the hippocampus (**Figure 2**) and whole embryo staining (**Figure 3**).

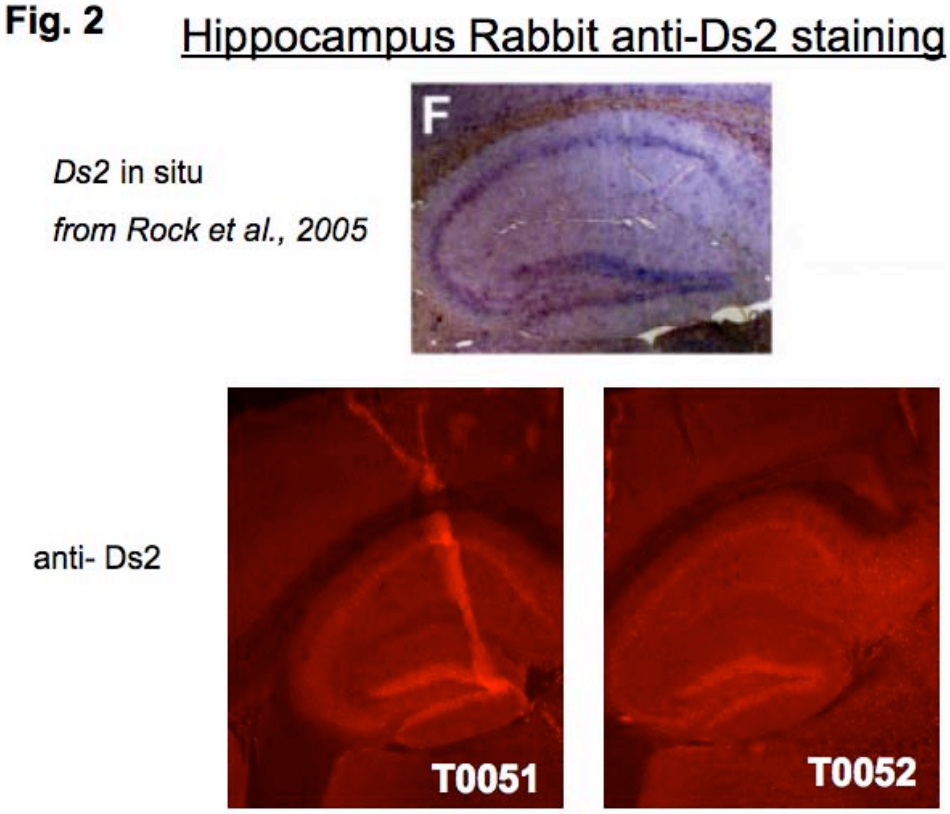

Figure 2. Validation of Ds2 antibodies. Top shows an in situ hybridization expression pattern of Ds2 in the hippocampus (taken from Rock et al., 2005). Bottom shows immunofluorescence of sections of the hippocampus, probes with antibodies to Ds2 (TO51 and TO52) that were effective in detecting Ds2 protein in immunofluorescence and western blot analysis.

Fig. 3 E12.5 embryo anti-Ds2 staining

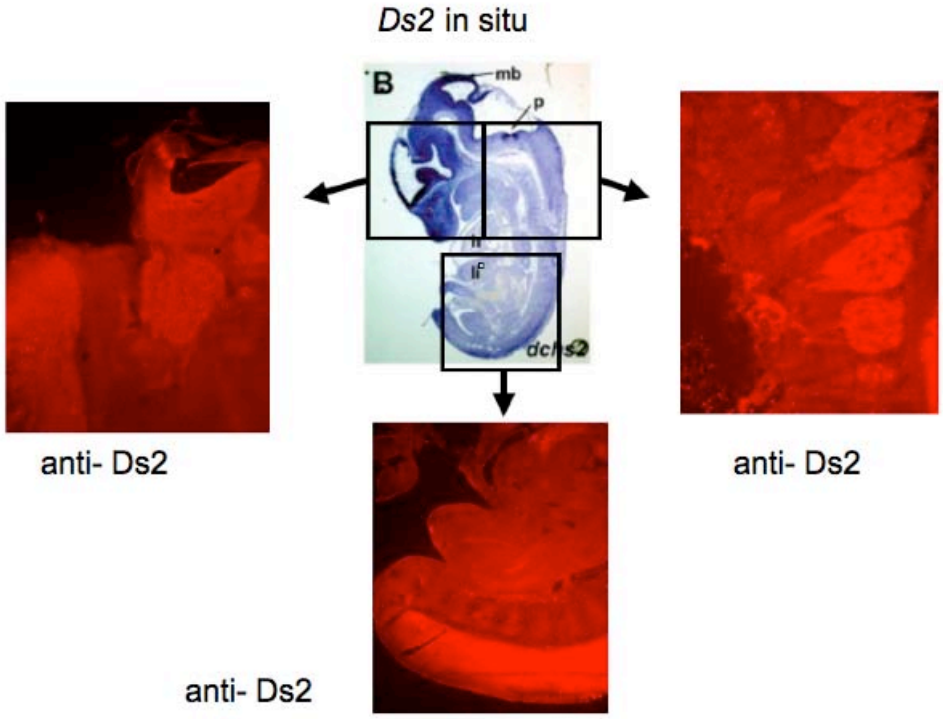

Figure 3. Immunofluorescence using specific Ds2 antibodies reveals the same pattern as published images of Ds2 by in situ hybridization (taken from Rock et al., 2005).

B) Test by RT-PCR if increased Ds2 expression validates in tumour samples. To determine if Ds2 mRNA is increased in tumour samples, we have used RT-PCR in collaboration with the laboratory of Dr. Irene Andrulis. Our initial studies assayed cell lines to optimize PCR conditions. Expression was normalized to the housekeeping gene, *hypoxanthine phosphoribosyltransferase 1* (HPRT1). We found that most cell lines express little or no Ds2 mRNA, although there was detectable expression in some cells such as SKOV3. The highest expression detected in cell lines was found in NTERA-2 cells (**Figure 4** and data not shown). Preliminary analysis of a panel of tumor samples revealed that most had low levels, on the order of most cell lines, however a few have very high levels, even higher than in NTERA-2 cells **(Figure 5**). This suggests that some recurrent tumors may indeed express unusually high levels of Ds2, however the finding thus far are not statistically significant, and more tumours must be assayed to reach a definitive conclusion.

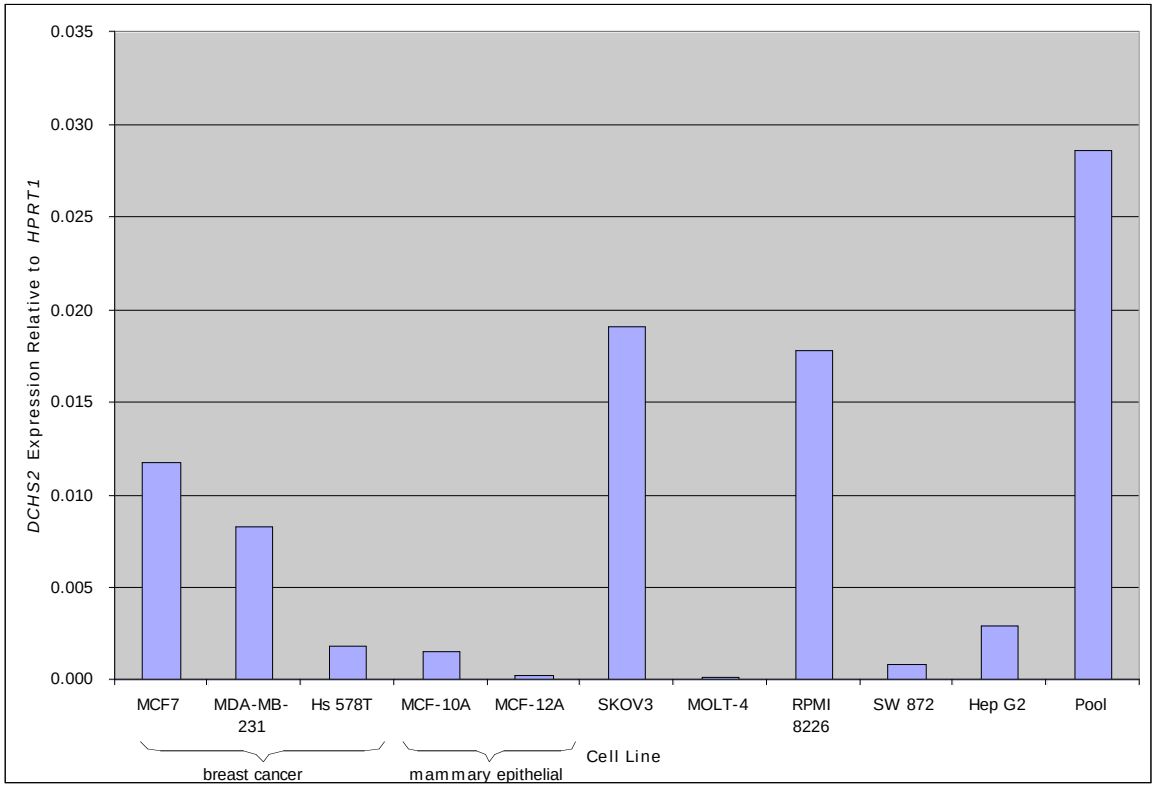

Figure 4. Quantifying expression of *dachsous 2* (DCHS2) in cell lines and a pool of 13 cell lines, using the real-time reverse-transcription polymerase chain reaction. Expression was normalized to the housekeeping gene HPRT1.

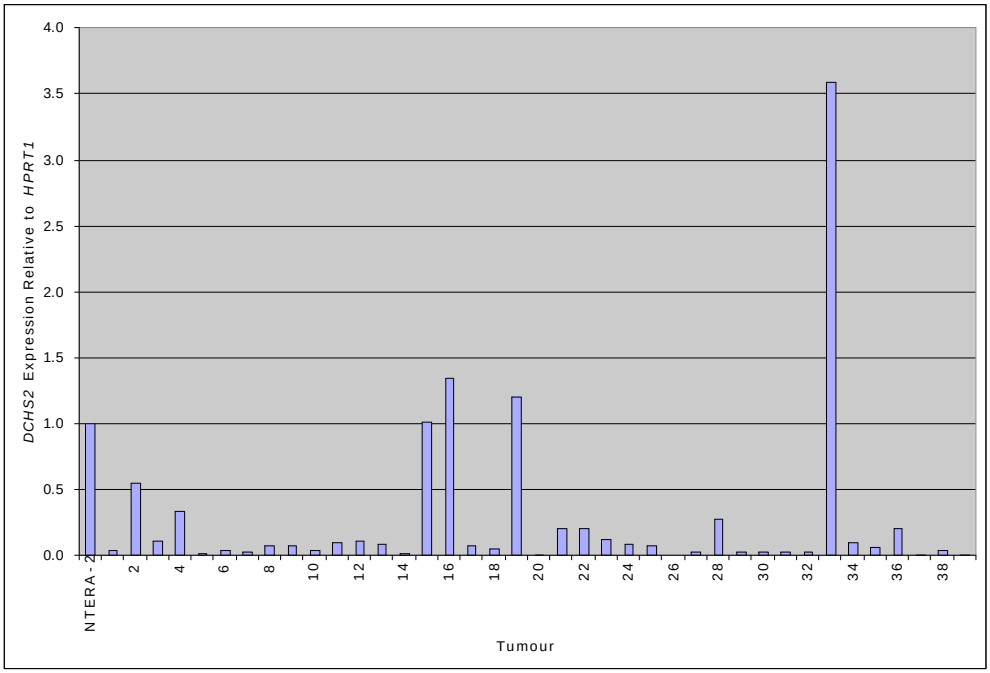

Figure 5. Analysis of Ds2 expression in tumours from ANN patients. Expression was low in most tumours but was very high in a subset of recurrent tumors.

C) Test specificity of Ds2 antibody and optimize staining of tissue sections
While the staining pattern of Ds2 in wild type mouse embryos is very encouraging, as it closely reflects the *in situ* pattern, the ideal control would be to assess staining in a Ds2 mutant. We have entered into a collaboration with the group of Dr. Francoise Helmbacher, Marseille, France, who has generated a Ds2 mutant. We will shortly be obtaining the Ds2 mutant mice from Dr. Helmbacher, and will conduct our staining in Ds2 mutants to provide genetic validation of the antibodies. In parallel we have been optimizing staining for paraffin sections, and have obtained the best results with use of "decloaking" reagents.

Task 2. ***Determine if the Hippo or PCP pathway is activated with increased Ds2 levels.***

1.A) Generate or obtain antibodies against Yap, MST1/2 and generate *in situ* probes for *fjx1. B)* Optimize staining on tissue sections. We have obtained antibodies to Yap and MST1/2 from commercial sources, and have generated *in situ* probes for *fjx1*. We have optimized staining for these reagents and are ready to commence screening of tissue microarrays. The analysis of the microarrays has not yet been conducted, as it is more efficient and reproducible to screen all our our reagents at the same time. As noted in Task1, the generation of specific antibodies to Ds2 was not trivial. However, now that we have generated good, highly specific antibodies to Ds2, we intend to commence analysis of the tissue microarrays immediately, using antibodies to Ds2, YAP, MST1 and MST2. We are also attempting to generate antibodies to Fjx1.
2, A) Screen tissue microarrays with antibodies to Yap, Mst1/2,fjx1
B) Assess if Ds2 overexpressing tumours are correlated with tumours with altered PCP or Hippo activation. We are just about to commence these studies, as described above.

Task 3. ***Ascertain if overexpression of Ds2 affects tumour incidence or metastasis in mouse models of breast cancer.***
A) Generate construct for transgenic analysis of Ds2 function. No functional studies have yet been conducted on Ds2 in mammals, and a full-length Ds2 cDNA was not available. We initiated our cloning of Ds2 cDNA by conducting PCR from embryonic mouse cDNA libraries. We designed our PCR primers based on the available reference sequence predicted from the mouse genome. We generated embryonic cDNA libraries from E17.5 embryos. The full length Ds2 cDNA was predicted to be 8 kb, and we amplified the cDNA in several pieces, as we were unable to amplify the predicted full-length transcript. Sequencing of this transcript revealed several portions that were missing, in comparison to the predicted sequence. Examination of the mouse and human genome indicated that the missing sequences corresponded to predicted Ds2 exons. This indicated that there was unsuspected complexity in the splicing of this transcript. In

collaboration with Dr. Lisa Goodrich, we have isolated a number of independent Ds2 transcripts. This raises the question of which is the appropriate Ds2 cDNA to use for transgenic overexpression to assay the effects on the PCP and Hippo pathway in mice. All of the transcripts contain exon 25, which was on the microarray that detected increased *Ds2* in tumours from woman with recurrent breast cancer, therefore this cannot be used to discriminate among these alternate splice forms. To ensure that we have chosen a functional splice form, we will test each cDNA by transient transfection into cells that express Fat4. Functional Ds2 should be recruited to cell–cell junctions, upon interaction with Fat4. We will be able to detect accumulation of Ds2 using the antibodies we have generated in task 1. Once we have verified that the various Ds2 isoforms are functional we will continue to testing induction of Ds2 with Doxycline in previously described aim 3B&C (*B)* Select ES cells that overexpress Ds2 under the control of Doxycline. *C)* Test induction of Ds2 protein levels in cell culture, using RT-PCR and western blot analysis)

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KEY RESEARCH ACCOMPLISHMENTS:

- Determination of novel splice forms of Ds2
- Generation and validation of antibodies specific to Ds2
- Cloning of full-length Ds2 and subcloning into tetracycline-responsive vectors
- Identification of high levels of Ds2 in a subset of tumors.

REPORTABLE OUTCOMES
-Specific Antibodies to Ds2

CONCLUSION:

In previous studies we identified increased levels of *Ds2* mRNA as a predictor of recurrence in woman with axillary node negative breast cancer. This lead to the hypothesis that increased levels of Ds2 mRNA and protein may provide a biomarker for recurrent breast cancer. To test this hypothesis, we have generated specific antibodies to Ds2, optimized Ds2 staining on paraffin sections, cloned full length Ds2 cDNAs and obtained antibodies and in situ probes to components of the mammalian Hippo and Planar Cell Polarity Pathway . These tools will allow us to test if increases in Ds2 is a good biomarker for recurrent breast cancer. In addition we will use these tools to determine the signal transduction pathways that are disrupted when Ds2 is overexpressed. These tools will also allow us to generate mice overexpressing Ds2, to determine if overexpession of Ds2 is a causal factor in breast cancer recurrence.

APPENDICES: N/A

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